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Robert Harold Schwarzhoff
Iowa State University

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Studies on the mechanism of swarm-cell formation in *Proteus mirabilis*

by

Robert Harold Schwarzhoff

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INTRODUCTION

The behavioral response known as swarming has been recognized and associated with the genus Proteus since the first description of the organism by Hauser (1885) nearly a century ago. The response can be described briefly as the movement of highly elongated and flagellated "swarm cells" across the surface of a solid medium in periodic cycles of movement and consolidation. During consolidation, the swarm cells divide to produce short cells that grow and divide for a period of time, then "differentiate" to form another generation of swarm cells.

While the phenomenon has been recognized for many years, we still know very little about the mechanisms responsible for controlling the response. Many theories have been proposed, but in general they have been supported by little or no experimental evidence. Recent evidence from this laboratory indicates that a critical event in swarming is the formation of swarm cells, and their actual movement does not seem to be related to the factors that bring about differentiation.

This study was therefore initiated as an attempt to define the mechanism of swarm-cell formation in Proteus mirabilis. Previous theories were based largely on the premise that swarm cells are involutionary forms, that is, they are defective cells, induced by a toxic environment. This interpretation seems to have developed because of the similarities between swarm cells and the filamentous forms produced by the action of numerous toxic compounds. However, this interpretation does not account

for the development of excessive numbers of flagella or the very active movement of swarm cells on a solid surface.

This study is an attempt to explain the formation of swarm cells as a specific, genetically controlled response that is triggered by changes in the cell environment. As such, swarm-cell formation may represent a unique form of morphogenesis that endows the organism with a distinct survival advantage, and it is the purpose of this dissertation to examine the potential role of catabolite repression in controlling this response.

LITERATURE REVIEW

Swarming

A significant amount of information has accumulated on the morphological events associated with swarming and the physical and chemical factors that influence it. Only recently, however, has it become apparent that the process can be resolved into specific phases that can be studied independently. The recent studies from this laboratory (Williams et al., 1976) have provided, for the first time, experimental evidence that the phenomenon can be divided into three distinct stages: The formation of swarm cells, their movement across the agar surface, and their subsequent reversion back to short forms.

The formation of swarm cells has been well characterized morphologically and appears to involve at least two specific events: Cell elongation and stimulated flagella synthesis. Probably the best description of these changes comes from the studies by Hoeniger (1964, 1965, 1966). In broth culture, P. mirabilis normally occurs as short rods about 0.6 μm wide and 1 to 2 μm long, possessing 1 to 10 flagella per cell. On a suitable agar medium, these short cells enlarge slightly, forming rods about 0.8 μm wide and 2 to 4 μm long. These cells undergo normal growth and division for a period of time (normally 2 to 3 hours), then those cells near the perimeter of the colony begin to elongate, forming filamentous cells about 0.7 μm wide and 20 to 80 μm long. Associated with elongation is a dramatic increase in flagellation so that a normal swarm cell may have 500 to 5000 flagella. In addition to the increase in the number of flagella per cell, there are also increases

in the number of flagella per unit length of cell and the average length of the flagella.

Very little is known about the mechanisms that control these changes. Investigations by Hoeniger (1966) and Jones and Park (1967a) indicate that during the process of elongation, nuclear development continues normally so that "mature" swarm cells may have as many as 46 evenly-spaced nuclei (no nuclear equivalents) along the entire length of the cell. This observation is evidence that elongation is not caused by inhibition of DNA synthesis, and the report of Armitage et al. (1974) provides further evidence for this. They found that swarm cells, placed on a medium with nalidixic acid (an inhibitor of DNA synthesis), would divide to produce short cells, but these short cells would not grow and divide subsequently. It appears, therefore, that elongation is caused by an inhibition of septation, and that growth and DNA synthesis proceed normally.

It is clear that filamentous forms can be induced by a variety of chemical and physical treatments, and Hughes (1956, 1957) was convinced that swarm cells were formed by a similar mechanism. The report by Duguid and Wilkinson (1961) that septation was particularly sensitive to such treatments seems to be additional evidence for this. It has not been shown, however, that swarm cells are identical to chemically-induced long forms. In fact, the little evidence that is available indicates that they are not the same (Hoeniger, 1966).

Whereas the most obvious characteristic of swarm cells (at the light microscopic level) is their extreme length, the degree of

flagellation seems to be just as important. Unfortunately, this aspect has received little attention from investigators attempting to explain the mechanism of swarm-cell formation. Jones and Park (1967a, b) speculated that uncontrolled flagella synthesis caused cell elongation by depleting the intracellular pool of amino acids, but they had no explanation for the stimulation of flagella synthesis. In Escherichia coli, flagella synthesis is subject to catabolite repression by glucose (Adler and Templeton, 1967), and its repression can be overcome by the addition of exogenous cyclic adenosine 3',5'-monophosphate (cAMP) (Dobrogosz and Hamilton, 1971). That flagella synthesis in Proteus is also subject to catabolite repression has not yet been shown.

The movement of swarm cells across the agar surface appears to be a consequence of both cell elongation and extreme flagellation. Morrison and Scott (1966) compared swarm cells to galleys, with the flagella serving as oars to propel the cells, as groups or "rafts", across the agar surface. While their analogy was based solely on microscopic observations, it seems to be a useful one.

Movement may be facilitated by, or even dependent on, the production of an extracellular slime. Fuscoe (1973) believed that swarming was dependent on such a slime, but his conclusion was based solely on microscopic observations. More convincing evidence for the existence of a slime has come from the examination of glutaraldehyde-fixed preparations in the scanning electron microscope (VanderMolen and Williams, circa 1976).

Lominski and Lendrum (1947) believed that the movement of swarm cells was a negative-chemotactic response away from toxic metabolites, and their theory has received a great deal of acceptance. However, our results (Williams et al., 1976) contradict this interpretation and provide good evidence that the movement of swarm cells away from the primary colony is neither negative nor positive chemotaxis. Our conclusions were based on the following observations: Swarm cells, removed from one medium, would continue to swarm outward when placed on fresh medium or even on a non-nutrient medium with added detergents. Dialysis of a solid medium from beneath failed to prevent swarming. Cells of Proteus mirabilis failed to respond significantly in negative-chemotaxis assays. Mutants of P. mirabilis that had lost the ability to swarm were still able to respond in a positive-chemotaxis assay. Mutants of P. mirabilis that had lost the ability to respond chemotactically were still able to swarm. These results provide convincing evidence that the outward movement of swarm cells is a nonchemotactic response, and it may be simply a consequence of their ability to move across the surface of a solid medium.

The period of consolidation, when swarm cells divide to produce short forms, is poorly understood and has received little attention from investigators. There is some controversy among investigators about the viability of swarm cells. Hughes (1957) believed that only a small fraction of them remained viable, but Hoeniger (1964) failed to detect any significant loss of viability. The mechanism that controls

consolidation is almost certainly related to that that controls the formation of swarm cells; so any hypothesis to explain one, should likewise explain the other.

A significant amount of information on swarming that is in the literature has not been discussed here; most of it concerns chemical and physical factors that affect the process. Unfortunately, we know so little about the actual mechanisms involved that this information is difficult to evaluate. For additional information on the subject, the review by Smith (1972) is a good source. There are a few characteristics that seem to be important and specific enough that they should be described at least briefly.

Perhaps the most unique characteristic of the phenomenon is that swarm-cell formation does not normally occur in broth; it is unique to a solid surface. Whereas there is evidence that agar contains a fraction that stimulates swarming (Jeffries and Rogers, 1968), it does not appear to be absolutely essential because swarming will occur on a gelatin-solidified medium (Hauser, 1885).

The process of swarming is very sensitive to the salt concentration of the medium, and while sodium chloride is most commonly used to satisfy this requirement, a number of other compounds will also work (Naylor, 1964; Schneierson, 1961). The requirement does not appear to be simply osmotic because dulcitol will satisfy or overcome the requirement but glucose will not (Naylor, 1964).

A large number of drugs, metabolic inhibitors, and inorganic compounds will inhibit swarming, but most of them appear to exert

their effect by inhibiting growth or motility. One that seems to be unique in its specificity is p-nitrophenylglycerol because, at physiologically active concentrations (0.1 to 0.2 mM), it affects neither growth nor motility but is a strong antiswarm agent (Williams, 1973). It appears to specifically inhibit the formation of swarm cells but does not inhibit their movement (Hoffman, 1974).

Catabolite repression

In order to evaluate the potential role of catabolite repression in the swarming response of Proteus, it is essential that the available information on catabolite repression be reviewed. The term, catabolite repression, was coined by Magasanik (1961) to describe the phenomenon, previously known as the "glucose effect". It is worthwhile to summarize some of the information he reviewed in that paper because it illustrates the general considerations important in understanding the phenomenon of catabolite repression, especially in evaluating its potential role in swarming. Originally, the glucose effect was defined as the inhibition of synthesis of certain enzymes by glucose, but as additional evidence accumulated, it became clear that the glucose effect was only one example of a more general phenomenon. Whereas glucose appeared to be most effective (in those organisms studied) in repressing the synthesis of a large number of enzymes, other substrates produced a similar effect. Repressible enzymes possessed the common feature of supplying catabolites which could be produced more readily or more efficiently from an

alternate source. This was the concept that led Magasanik to propose the term, catabolite repression, to describe the phenomenon.

Accordingly, regulation of enzyme synthesis by catabolite repression would provide an organism with a distinct survival advantage in at least two ways: An organism growing in the presence of two substrates, both capable of supplying the same catabolites, would synthesize the enzymes necessary to utilize only one, therefore, sparing the cell the expense of synthesizing additional unnecessary protein. A second advantage would involve the repression of enzymes that degrade essential compounds (such as amino acids) to catabolites that could be supplied by more appropriate substrates. This would be particularly important in cases where the organism was unable to synthesize that compound de novo.

Since Magasanik described the concept of catabolite repression, considerably more information has accumulated on additional proteins subject to repression, and on the mechanisms involved in mediating this type of regulation (Rickenberg, 1974). While this information extends and modifies the concept somewhat, the general considerations of its overall value remain valid. In general, the proteins regulated by catabolite repression are not required by the cell under all conditions. It is a dynamic process that allows an organism to expend a minimal amount of energy when growing under specific conditions yet retain its ability to adapt readily to changing conditions.

It is now generally accepted that the synthesis of proteins, subject to catabolite repression, is regulated by the intracellular

concentration of cAMP, and substrates like glucose that repress these proteins lower the concentration of this nucleotide (Rickenberg, 1974). Preliminary evidence for this mechanism came from the findings that exogenous cAMP would overcome the repression of a number of inducible enzymes (De Crombrugghe et al., 1969; Pastan and Perlman, 1968; Perlman and Pastan, 1968). Indirect support has also come from measurements of cAMP in cells growing under different culture conditions. The results from a number of investigators indicate that the intracellular cAMP concentration is higher in cultures growing on substrates that support slow growth than it is in cultures growing on substrates that support rapid growth (Buettner et al., 1973; Pastan and Perlman, 1970; Peterkofsky and Gazdar, 1971). In addition, Buettner et al. (1973) were able to show a direct correlation between the level of cAMP and the specific activity of β-galactosidase, an enzyme subject to catabolite repression. Finally, it appears that when an organism exhausts the supply of a readily-utilizable substrate such as glucose, the level of cAMP increases dramatically (Makman and Sutherland, 1965; Peterkofsky and Gazdar, 1971).

While these results appear to be reasonably convincing, they must be considered with caution because there are some serious criticisms. The ability of cAMP to overcome catabolite repression is dependent on the substrate that causes repression; whereas cAMP reverses the repression of β-galactosidase by glucose, it has limited ability to overcome repression by glucose-6-phosphate or glucose plus gluconate (Goldenbaum and Dobrogosz, 1968). A second consideration applies to

measurements of intracellular-cAMP concentrations. Because these levels are very sensitive to the suspending medium and can change very rapidly, their measurement must be carefully performed, and the results require critical interpretation (Rickenberg, 1974).

The most definitive evidence for the role of cAMP in catabolite repression has come from in vitro experiments designed to identify the specific requirements for expression of catabolite-sensitive genes. In the first experiments of this type, Zubay's group used cell-free extracts and showed that cAMP stimulated DNA-dependent synthesis of β-galactosidase (Chambers and Zubay, 1969; Zubay et al., 1970). Because of the complexity of the system, however, it was difficult to identify which step in gene expression was stimulated by the cAMP. By using more highly refined systems, investigators have identified the cAMP-dependent step in gene expression at the level of transcription (De Crombrugghe et al., 1970, 1971; Eron et al., 1971; Nissley et al., 1971). In the experiments of De Crombrugghe et al. (1970, 1971) DNA from a transducing phage (λh80dlac) was used as template, and transcription was assayed by measuring hybridization of the mRNA with the appropriate phage DNA. Transcription was found to be dependent on the presence of cAMP, a cAMP receptor protein (CRP), and RNA polymerase. The results from these and other investigations indicate that cAMP binds to the receptor protein, and this complex then interacts with the bacterial DNA (apparently at promotor sites) and facilitates binding and subsequent transcription by the RNA polymerase (Rickenberg, 1974).

According to this model, expression of catabolite-sensitive genes could be regulated by the intracellular level of cAMP that is available for binding, with the receptor protein, to the chromosome. Whereas the enzymes responsible for the formation (adenyl cyclase) and degradation (cAMP phosphodiesterase) of cAMP and the receptor protein itself have been well characterized, little is known about how they interact, *in vivo*, to control the level of cAMP or the "active" cAMP-CRP complex.

Adenyl cyclase, the enzyme that converts ATP to cAMP, has been purified from *E. coli* by Tao and Lipmann (1969); it has a molecular weight of 110,000 and requires Mg^{2+} for activity. Ide (1969) found that the activity of the purified enzyme was stimulated by some compounds and inhibited by others, but these effects were observed only when the compounds were used at extremely high concentrations. More recently, Peterkofsky and Gazdar (1974) employed an *in vivo* system and found that glucose strongly inhibited the activity of the enzyme. A number of investigators have isolated adenyl cyclase-deficient mutants, and this approach has provided much of the information on what cell functions are dependent on cAMP (Kumar, 1976; Perlman and Pastan, 1969; Yokota and Kuwahara, 1974). In general, these mutants are unable to utilize a number of carbohydrates, are nonmotile, may lack specific somatic antigens, have increased generation times, have altered cell morphologies, and are more sensitive to a number of chemical and physical agents. Exogenous cAMP overcomes this mutation and restores most of the normal cell functions.

cAMP phosphodiesterase, the enzyme that degrades cAMP to adenosine 5'-monophosphate (5'AMP), has been purified from E. coli by Nielsen et al. (1973). It has a molecular weight of 30,000 and requires Fe^{2+} and a reducing compound for activity, and there is some evidence that its activity may be controlled by the redox potential of the cell. However, it does not appear to play a major role in regulating the cAMP concentration of the cell, because a mutant that lacked the phosphodiesterase still demonstrated levels of cAMP that depended on the carbon source being utilized (Buettner et al., 1973).

The third protein, unique to the cAMP system, is the cAMP receptor protein. This protein, also called the catabolite gene activator protein, was first isolated on the basis of its ability to stimulate DNA-directed in vitro synthesis of β-galactosidase (Zubay et al., 1970). Subsequently, Pastan and his collaborators isolated and purified the protein on the basis of its ability to bind tritiated cAMP (Emmer et al., 1970). According to Riggs et al. (1971), the receptor protein from E. coli is a dimer with subunits of 22,000 daltons each. The purified protein has significant affinity for DNA, and this affinity is enhanced by cAMP. Cyclic guanosine 3',5'-monophosphate (cGMP) competes with cAMP for binding with the protein, and the resulting complex neither binds to DNA nor stimulates in vitro synthesis of β-galactosidase and tryptophanase synthesis. Mutants, defective in the receptor protein, have been isolated from a number of genera; they are similar to adenyl cyclase mutants, but exogenous cAMP fails to

overcome the block (Artman and Werthamer, 1974; Iuchi et al., 1975; Kumar, 1976).

Despite the abundance of information available on the cAMP system, the primary question of what controls the level of cAMP in the cell remains unanswered. Whereas there is evidence that the adenyl cyclase and phosphodiesterase play roles in the regulation of this concentration, fluctuations in the activities of these enzymes alone cannot explain the observed changes in cAMP levels (Rickenberg, 1974). An alternative mechanism that has received attention from several investigators involves the rapid release or transport of cAMP from the cell. During some of the earliest investigations in this field, Makman and Sutherland (1965) observed that glucose, added to cultures of starved cells, stimulated the release of cAMP into the medium. The recent reports of Seto et al. (1975a, b) suggest that the cAMP level in the cells is controlled by the interaction of more than one mechanism; rapid changes are probably controlled by the rate of release of cAMP, but more stable levels are probably controlled by the relative activities of the adenyl cyclase and phosphodiesterase. There are other potential control mechanisms in the cAMP system that have not received much attention from investigators. They include competition by cGMP for binding to the receptor protein (Haggerty and Schleif, 1975), and changes in the level or binding activity of the receptor protein itself.

It should be noted that nearly all of our present knowledge of the cAMP system and catabolite repression has been obtained from E. coli, and while one would expect the systems in P. mirabilis to be similar, the lack of available information requires some caution in comparing the two organisms.

MATERIALS AND METHODS

Organism

The wild-type Proteus mirabilis strain used in this study was obtained from the culture collection of the Department of Bacteriology, Iowa State University, and will be referred to as IM47. The nonswarming mutants were isolated following exposure to N-methyl-N'-nitro-N-nitrosoguanidine and have been previously described (Williams et al., 1976). Stock cultures were maintained according to the procedure of Chance (1963). Nutrient agar slants were uniformly inoculated and incubated for 16 hours, then the cells were washed from the surface of the agar with 5 ml of sterile 1% NaCl, and the suspension was transferred to a sterile test tube and refrigerated. For routine experiments, a loopful of the refrigerated suspension was transferred to a tube of an appropriate broth medium and incubated overnight. With this procedure, no significant losses in viability (as estimated by growth of an inoculum) were detected with storage for as long as six months. After that period, the cultures were routinely recloned, and fresh stock cultures were prepared.

Media

Tryptose broth contained 1% tryptose (Difco) and 0.5% NaCl, and tryptose agar was prepared by adding 1.5% agar (B.B.L., Becton Dickinson) to the broth. Tryptose soft agar was used to test for chemotaxis and was prepared by adding 0.2% agar to tryptose broth. Nutrient broth was

obtained from Gibco and nutrient agar was prepared by adding 1.5% agar to the nutrient broth.

A basal salts and vitamin mixture, modified from the one described by Jones and Park (1967b), was used for the preparation of synthetic media. It was prepared from five solutions: Solution A contained per liter, K_2HPO_4, 24.9 g; KH_2PO_4, 10.7 g; $(NH_4)_2SO_4$, 1.2 g; and NH_4Cl, 1.2 g. Solution B contained 0.2 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ in 100 ml of 0.02 N HCl. Solution C contained 0.4 g of $MgSO_4 \cdot 7\ H_2O$ in 100 ml of water. Solution D contained 24.0 mg of nicotinic acid in 100 ml of water. Solution E contained 24.0 g of NaCl in 100 ml of water. Solution B was membrane filtered (0.22 μm pore size, Millipore); the remaining solutions were autoclaved for 15 minutes at 121 C.

The final medium contained 450 ml of solution A, 10 ml each of solutions B, C, and D, 20 ml of solution E, and 500 ml of water containing the particular carbon and energy source at the appropriate concentration (autoclaved separately). For solid media, an agar solution at the appropriate concentration was prepared and added as part of the 500 ml. All solutions were prepared with deionized water and were stored at room temperature. The final pH of the medium was 7.0.

Chemicals

Cyclic adenosine 3',5'-monophosphate (cAMP), cyclic guanosine 3',5'-monophosphate (cGMP), guanosine 3'-monophosphate (3'-GMP), guanosine 5'-monophosphate (5'-GMP), ribose, and methylglyoxal were obtained from Sigma Chemical Co., St. Louis, Mo. Adenosine

5'-monophosphate (5'-AMP) and adenosine were obtained from Schwarz Labs Inc., Jamaica, N.Y. Adenine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. 2,4-Dinitrophenylhydrazine (DNPH) and ethylenediaminetetraacetic acid (EDTA) were obtained from Eastman Kodak Co., Rochester, N.Y. Kinetin (6-furfurylaminopurine) was obtained from Calbiochem, San Diego, Cal. Cyclic adenosine-8-^{3}H-3',5'-monophosphate (^{3}H-cAMP, 13.7 Ci/mmole) was obtained from I.C.N. Corp., Irvine, Cal. The 2,5-diphenyloxazole (PPO) was obtained from Beckman Instruments Inc., Fullerton, Cal., and naphthalene was obtained from Matheson, Coleman, and Bell Manufacturing Chemicals, Norwood, Ohio.

The effect of nucleotides and related compounds on swarming and swarm-cell formation

cAMP, 3'-AMP, 5'-AMP, adenine, adenosine, ribose, potassium phosphate (pH 7.0), cGMP, 3'-GMP, 5'-GMP, kinetin, and pantoyl lactone were incorporated at varying concentrations in tryptose agar to determine their effects on the time of onset and extent of swarming. The acidic nucleotides were neutralized with concentrated NaOH prior to their addition, and all compounds were membrane filtered and added aseptically to the molten tryptose agar at 45 C. The plates were poured and then dried overnight at 35 C to remove excess surface moisture. Each plate was inoculated centrally with a loopful of an overnight, tryptose-broth culture of IM47. The plates were incubated at 35 C and examined microscopically to determine the time of onset of swarming. The effect

of exogenous cAMP on swarming of nonswarming mutants was examined in a similar manner.

In addition, the effect of exogenous cAMP on cells in tryptose broth was examined. Ten-ml volumes of a tryptose-broth culture of IM47, in the early-exponential phase of growth (turbidimetrically determined), were distributed to 125-ml Erlenmeyer flasks and incubated at 35 C on a rotary shaker. After 30 minutes of incubation, different amounts of cAMP (prepared and neutralized in tryptose broth) were added to the individual flasks, and the cultures were examined microscopically at 30-minute intervals to detect any changes in morphology or motility.

Carbon-source utilization by IM47 and the nonswarming mutants

The ability to use different substrates as the sole source of carbon and energy was determined for IM47 and several nonswarming mutants derived from it. Synthetic agar media, with each carbon source at a final concentration of 0.5%, were prepared and dispensed into petri dishes. The plates were dried overnight and inoculated with a loopful of an overnight culture (in tryptose broth) as a spot inoculum. Growth was assessed, qualitatively, as the ability of the organism to form a confluent colony in 48 hours at 35 C. The temperature-sensitive, nonswarming mutants were examined for growth at both 25 C and 35 C. Fermentation broths (nutrient broth with 1% of the carbon source and 0.016 g of brom thymol blue per liter) were used to distinguish mutants, unable to use any of the carbon sources, from auxotrophic mutants.

The effect of exogenous cAMP on growth of IM47 and the nonswarming mutants on different carbon sources

The growth responses of IM47 and the nonswarming mutants on synthetic agar media with added cAMP were determined qualitatively. The carbon sources that supported growth of IM47 were used at a final concentration of 0.5% and the cAMP was added to a final concentration of 5 mM. The plates were spot inoculated with 12-hour cultures in synthetic broth containing 0.25% glyerol as the carbon source. The plates were incubated at 35 C and examined visually at 12-hour intervals for confluent-colony formation.

A number of experiments was performed to examine, quantitatively, the growth of IM47 and one of the nonswarming mutants, Nsw109, on synthetic media containing cAMP. In broth cultures, growth was measured by determining viable cell numbers in samples drawn at periodic intervals. To measure growth on an agar medium, plates were spot inoculated with 5-µl volumes of a broth culture, and at periodic intervals, the cells in a single colony were washed from the surface, quantitatively diluted, and plated on nutrient agar. The efficiency of this procedure for recovering cells from the agar surface was determined by comparing the viable cell number in the broth inoculum with the viable cell number determined on a freshly inoculated plate. Also, the viable cell number from the second washing of a colony was compared with that determined on the first washing of the same colony.

Identification and assay of methylglyoxal

Methylglyoxal was identified and assayed as its dinitrophenylhydrazine derivative according to the procedures of Freedberg et al. (1971) and Ackerman et al. (1974). The phenylhydrazones were formed by incubating 1 ml of sample (containing 1 to 10 µg/ml of methylglyoxal) with 0.3 ml of DNPH reagent (0.1% [w/v] DNPH in 2 N HCl) for 15 minutes at 30 C. For measurement, 1.7 ml of 2.5 N NaOH was added to the reaction mixture and this was incubated for 15 minutes at room temperature (about 25 C). The absorbance of the sample was then determined at 550 nm with a Spectronic 20 colorimeter (Bausch and Lomb) and compared with values from a standard curve prepared with authentic methylglyoxal. Samples were routinely examined using thin-layer chromatography to confirm the presence of methylglyoxal. For chromatography, the phenylhydrazones were extracted from the initial reaction mixture (sample plus DNPH reagent) with 1 ml of ethyl acetate, and plates of silica gel (250 µm layer) were spotted with 5 to 20 µl of the extract. The chromatograms were developed in the ascending direction with benzene-ethyl acetate (20:1), and the phenylhydrazones were detected by spraying with a 10% (w/v) solution of NaOH in 60% (v/v) ethanol.

To test for methylglyoxal production by organisms on a solid medium, the agar supporting the colony was cut out, placed in 1 ml of water and vigorously mixed with a vortex mixer. This preparation was then treated as described above.

A resting-cell assay was developed to quantify the amount of methylglyoxal produced by cells, grown under different culture

conditions. Cells, harvested from agar plates (uniformly inoculated) by washing, and cells from broth cultures were washed twice by centrifugation and resuspended in water. The final cell suspensions were adjusted to an absorbance of 1.0 at 540 nm. The assay mixtures (6.0 ml final volume) contained 1.5 ml of the cell suspension, glycerol at a final concentration of 1 mg per ml, and potassium phosphate buffer (pH 7.0) at a final concentration of 0.05 M. These mixtures, along with control mixtures, were incubated at 35 C, and at periodic intervals, samples were removed and assayed for methylglyoxal by the procedures described previously.

cAMP binding protein assay

An assay for the level or activity of the cAMP receptor protein was developed according to the procedures of Anderson et al. (1971) and Iuchi et al. (1975). It is based on the ability of the receptor protein, in a crude extract, to bind tritiated cAMP. The extract was prepared from cells in the late logarithmic phase of growth that were harvested and washed twice in dialysis buffer containing 0.05 mM EDTA, 3.5 mM 2-mercaptoethanol, and 10 mM potassium phosphate buffer (pH 7.6). The final cell pellet was resuspended in a small amount of buffer and passed twice through a French pressure cell (Aminco) at 18,000 to 20,000 p.s.i. The crude extract was centrifuged at 20,000 x g for 90 minutes, and the supernatant was removed and dialyzed for 16 hours at 4 C with three changes of buffer. The protein concentration in the extract was measured by the Biuret procedure (Gornall et al., 1949); bovine serum albumen (Gibco) was used for the standard curve.

The assays were performed by incubating, at 1 C for 5 minutes, mixtures containing 50 µl of stock buffer solution (40 mM 5'-AMP, 0.15 mM EDTA, 10.5 mM 2-mercaptoethanol, and 30 mM potassium phosphate buffer [pH 7.6]), 50 µl of water containing 100 pmoles of ^{3}H-cAMP (about 100,000 c.p.m. of radioactivity), and 50 µl of either water or 40 mM unlabeled cAMP. The reactions were started by adding the extract and terminated by adding 0.8 ml of saturated $(NH_4)_2SO_4$ solution. After 60 minutes of additional incubation, the mixtures were centrifuged at 5,000 x g for 20 minutes in a swinging-bucket rotor. The supernatant was removed and discarded, and each pellet was dissolved in 1.0 ml of water, transferred to a scintillation vial, and mixed with 10 ml of counting cocktail (5 g of PPO, 100 g of naphthalene, and dioxane to 1 liter). The vials were equilibrated overnight and counted the following day in a Beckman DPM-100 liquid scintillation spectrophotometer.

Binding activity was calculated by subtracting background counts (determined with reaction mixtures containing excess unlabeled cAMP) from sample counts, and dividing this value by the specific activity of the ^{3}H-cAMP (c.p.m. per pmole of cAMP). These values are, therefore, expressed as pmoles of cAMP bound per mg of protein in the crude extract.

Isolation of mutants defective in carbohydrate utilization

A mutagenesis and selection procedure was carried out to isolate mutants of IM47 that were defective in either adenyl cyclase or the cAMP receptor protein. Conditions for mutagenesis with ultraviolet

light were determined previously by Anderson (1975). Cells from a 5-hour culture of IM47 in tryptose broth were harvested by centrifugation, suspended in a 1% NaCl solution, and exposed to a 15-watt germicidal lamp (Champion G15T8) for 22 seconds at a dose rate of 12 ergs/mm^2/sec. The suspension was added to an equal volume of double-strength nutrient broth and incubated with shaking at 35 C for 90 minutes to allow for nuclear segregation. These cells were then harvested by centrifugation, washed once in water, and resuspended in a synthetic broth containing 0.5% trehalose. This culture was incubated with shaking at 35 C for 5 hours to allow for phenotypic expression, and then penicillin G (Eli Lilly) was added to a final concentration of 50 µg per ml and the culture was reincubated for 14 hours. The suspension was diluted and plated on either nutrient agar or a synthetic agar medium containing 0.5% glucose. Individual clones were subsequently tested for growth on glucose and trehalose by replica plating.

RESULTS

Effect of nucleotides and related compounds on swarming and swarm-cell formation

On tryptose agar at 35 C, IM47 normally initiated microscopically recognizable swarming between 3.5 and 4.5 hours following inoculation. However, this variation was only observed over extended periods of time and was probably caused by small changes in incubator temperature, batch-to-batch variation in medium composition, or even subtle changes in the organism itself. In a particular experiment, this value was quite constant, with the range for control plates, without any additions, rarely exceeding 10 minutes.

Table 1 is a summary of the results from four different experiments in which the effects of various compounds on the onset of swarming were examined. Of the compounds tested, cAMP was the most effective in stimulating premature swarming. At 5 and 10 mM concentrations, cAMP normally reduced the time between inoculation and onset of swarming by 20 to 40 minutes, and whereas this reduction seems small, it was reproducible from one experiment to another. The stimulatory effect of cAMP was evident from two additional parameters: The proportion of swarm cells present in the colony at the onset of swarming was considerably increased in the presence of cAMP, and the movement of the swarm band across the agar surface was continuous rather than periodic.

Of the remaining compounds tested, the 3'- and 5'-nucleotides also demonstrated reproducible activity, but they were not as effective as

Table 1. The effect of various compounds on the time of onset of swarming by P. mirabilis (IM47) on tryptose agar at 35 C

Compound	Trials	Time to onset of swarming (hours)[a] 5 mM	10 mM
cAMP	4	3.5	3.5
3'-AMP	4	3.8	3.7
5'-AMP	4	3.8	3.7
cGMP	2	4.1	4.2
3'-GMP	1	3.9	3.7
5'-GMP	1	3.9	3.8
Ribose	1	4.0	4.0
Adenine	1	4.3	4.5
Adenosine	1	4.0	4.0
Potassium phosphate	1	4.0	4.0
Kinetin	1	4.3	nt[b]
Pantoyl lactone	1	4.0	4.0

[a]Controls (with no additions) averaged 4.0 hours in 20 trials.

[b]Not tested.

cAMP. Adenine, cGMP, and kinetin inhibited swarming as indicated by the delay in the onset of swarming and distances that the swarm bands moved. Adenine appeared to inhibit growth of the organism as indicated by colony development.

The addition of cAMP to exponentially-growing cells in tryptose broth had no significant effect on cell morphology or motility (estimated by microscopic examination of hanging-drop preparations). The cAMP was tested at final concentrations between 0.001 and 100 mM. Similarly, preincubation of cells in the presence of cAMP and subsequent transfer to tryptose agar, with and without cAMP, did not significantly affect the time of onset of swarming that normally would have occurred.

When the nonswarming mutants were inoculated onto tryptose agar containing 5 mM cAMP, two of the mutants (Nsw203 and Nsw214) demonstrated a swarming response, but it was detectable only by microscopic examination. However, a revertant (designated Nsw214 sw rev) was isolated from Nsw214 that demonstrated a swarming response in the presence of cAMP that was similar to normal swarming. A chemotactic revertant (designated Nsw214 che rev), isolated from Nsw214 in tryptose soft agar, also demonstrated a similar response. Figures 1a and 1b illustrate the effect of increasing concentrations of cAMP on these two revertants, and the responses of these revertants and the original mutants to cAMP are summarized in Table 2. Finally, a revertant was isolated from Nsw214 sw rev that swarmed in the absence of cAMP. Even though this "double" revertant swarmed as actively as the wild type, it still retained some

Fig. 1. The effect of increasing concentrations of cAMP on swarming of Nsw214 sw rev (a) and Nsw214 che rev (b) on tryptose agar

Both plates contained 0, 1, 5, and 10 mM cAMP (clockwise from upper-left quadrant).

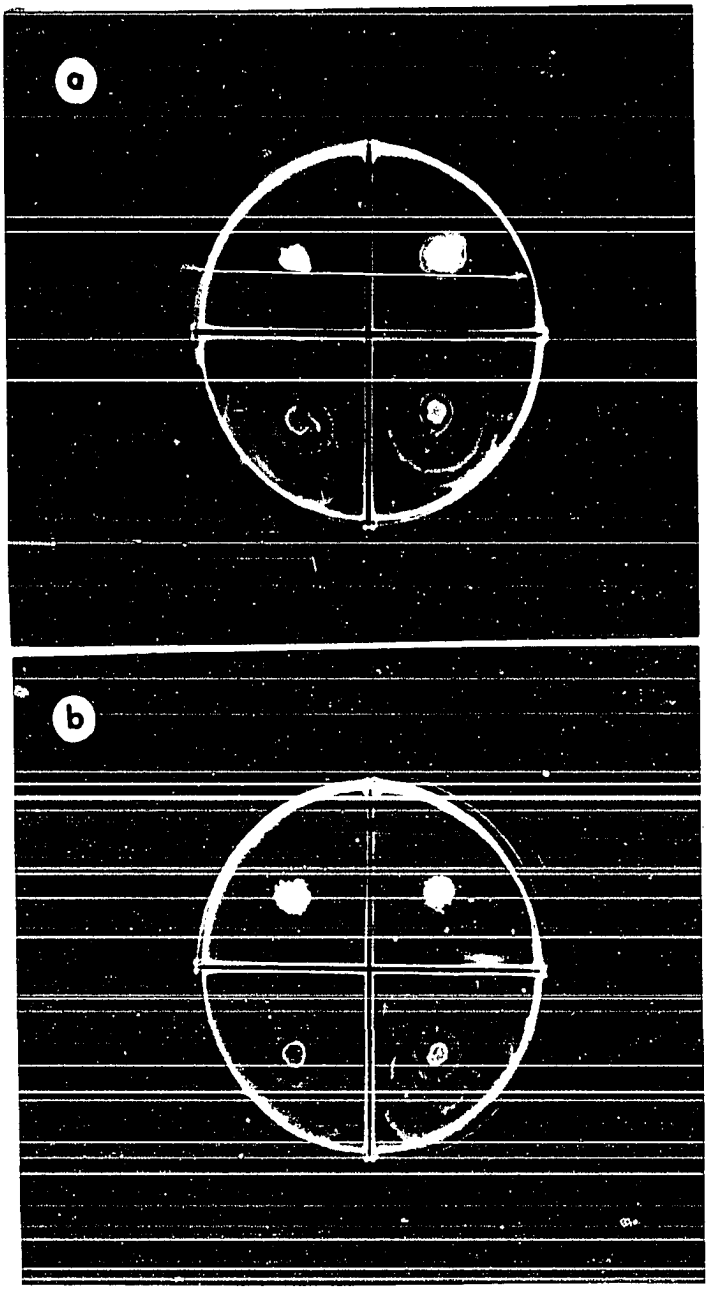

a
b

Table 2. The effect of cAMP and 5'-AMP on swarming and swarm-cell formation on tryptose agar in two nonswarming mutants and two revertants isolated from Nsw214

Strain	Without additions				With 5 mM cAMP		With 5 mM 5'-AMP	
	Mot[a]	Che[b]	SC[c]	Sw[d]	SC	Sw	SC	Sw
Nsw203	+	-	+	-	+	micro[e]	nt[f]	nt
Nsw214	+	-	-	-	+	micro	±	-
Nsw214 sw rev	+	-	-	-	+	normal	+	micro
Nsw214 che rev	+	+	+	micro	+	normal	+	macro[g]

[a]Mot - motility as determined by microscopic examination of wet mounts and by movement of cells in tryptose soft agar.

[b]Che - chemotaxis was assessed as the formation of chemotactic bands in tryptose soft agar.

[c]SC - swarm-cell formation as described by microscopic examination of cells.

[d]Sw - swarming.

[e]micro - swarming visible only by microscopic examination.

[f]nt - not tested.

[g]macro - swarming visible macroscopically but diameter of swarm bands less than normal.

characteristics of the original mutant (Nsw214): It failed to demonstrate normal chemotaxis in tryptose soft agar, and it also demonstrated the same pattern of carbon-source utilization as the original mutant (Nsw214).

Carbon-source utilization by IM47 and the nonswarming mutants

The following carbon sources supported growth of IM47 in a synthetic medium: Citrate, D-galactose, D-glucose, glycerol, DL-lactate, pyruvate, succinate, and trehalose. D-Mannose supported very slow growth, and the following carbon sources failed to support any growth: D-arabinose, D-gluconate, glycogen, D-fructose, maltose, D-mannitol, D-ribose, L-sorbose, sucrose, and D-xylose.

The nonswarming mutants were tested on synthetic agar media containing those carbon sources that support growth of IM47, and Table 3 summarizes their growth responses. Several mutants were unable to grow on any of the synthetic media, but all of these mutants were able to utilize galactose, glucose, glycerol, and trehalose in the fermentation broths. Utilization of the remaining carbon sources could not be determined with the fermentation broths, even for IM47. Either they failed to utilize these compounds under reduced oxygen concentrations or their utilization did not result in a pH change. Nsw101 and -110 swarmed at 25 C but not at 35 C, but the temperature of incubation had no effect on their carbon-source utilization. All of the revertants from Nsw214 that were described previously demonstrated the same pattern of carbon-source utilization as Nsw214. However, two additional revertants

Table 3. The growth responses of nonswarming mutants on synthetic agar media containing different substrates

Mutant	Growth								
	Citrate	Galactose	Glucose	Glycerol	Lactate	Mannose	Pyruvate	Succinate	Trehalose
Nsw101,-107 -109,-203 -206,-211 -215,-221 -227,-228 and IM47	+	+	+	+	+	+	+	+	+
Nsw104	+	+	+	+	-	+	-	+	+
Nsw110,-115 -122,-202 -231,-235	-	-	-	-	-	-	-	-	-
Nsw214	-	+	+	+	-	+	-	-	-

were isolated from Nsw214, one on citrate and the other on pyruvate. Both of these strains were able to utilize all of the carbon sources except trehalose and both failed to swarm. Exogenous cAMP failed to overcome the mutation(s) in Nsw214 that prevented it from using several of the carbon sources.

The effect of exogenous cAMP on growth of IM47 and the nonswarming mutants

Five mM cAMP significantly inhibited the growth of IM47 on synthetic agar media containing either galactose, glycerol, or trehalose. This effect was determined, qualitatively, by macroscopic examination of colonies on the agar surface after 24 hours of incubation. The nonswarming mutants were examined in this way, and of those that grew on the synthetic media, Nsw109, -221, and -228 were resistant to the inhibitory effect of cAMP on all three carbon sources. Figures 2a and 2b illustrate the appearance of IM47 and these three mutants on glycerol agar plates, with and without 5 mM cAMP, after 24 hours of incubation. The responses illustrated in Figure 2 are representative of the responses observed on galactose and trehalose agar plates. After 24 hours, however, IM47 overcame the inhibitory effect of cAMP on galactose and trehalose so that confluent growth was evident after 48 hours. On glycerol agar, inhibition by cAMP appeared to be more severe and essentially irreversible.

The growth responses of IM47 and Nsw109 were more carefully examined and Figure 3 illustrates, quantitatively, the effect of cAMP

Fig. 2. The growth responses of IM47, Nsw109, Nsw221, and Nsw228 (clockwise from upper-left position) on a glycerol (0.25%) agar medium (a) and the same medium with 5 mM cAMP

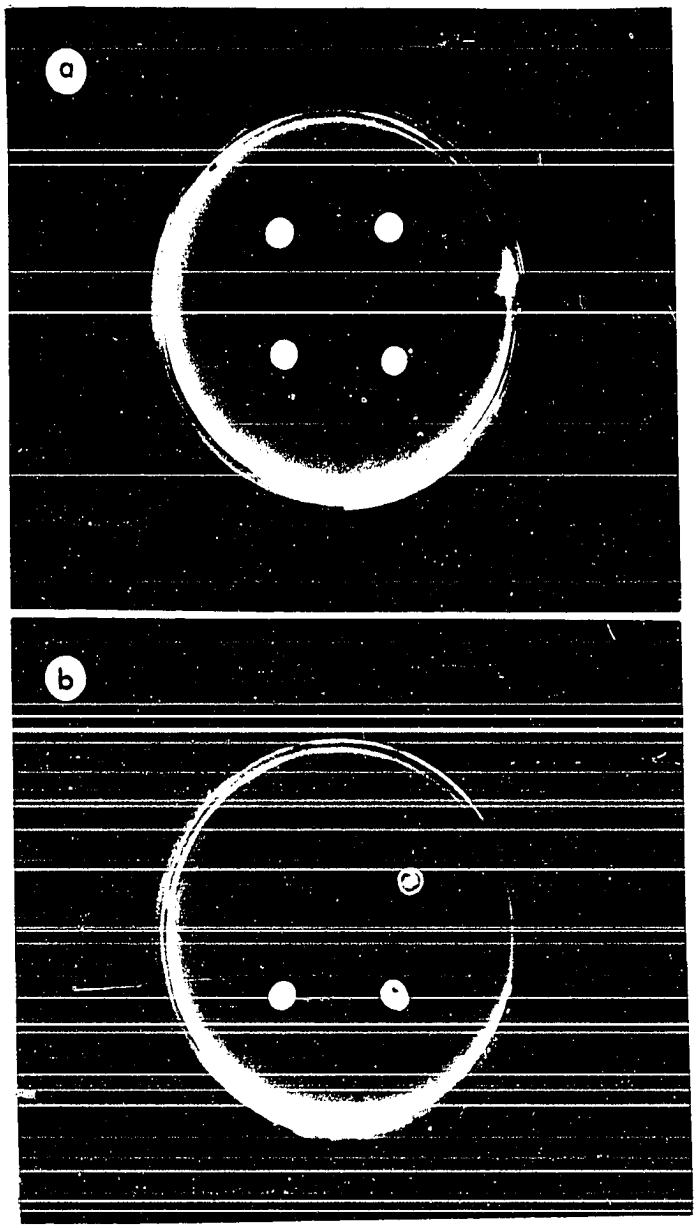
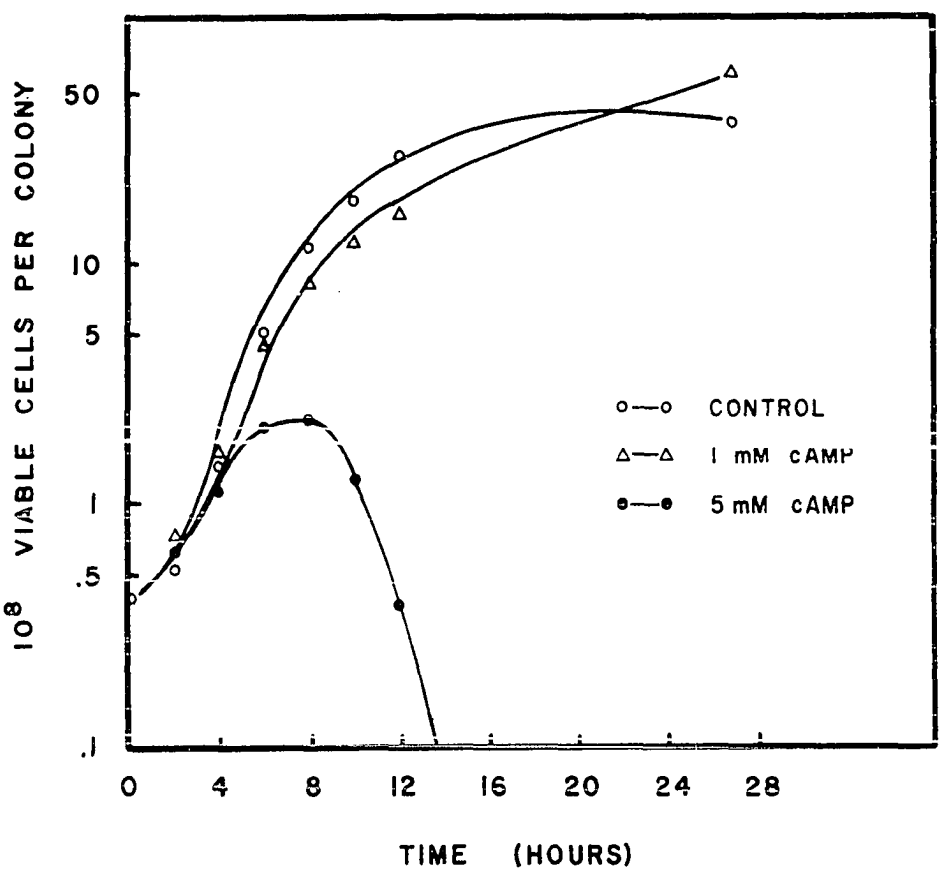

a
b

Fig. 3. The effect of exogenous cAMP upon growth of IM47 on a glycerol (0.25%) agar medium at 35 C


10^8 VIABLE CELLS PER COLONY
50
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.5
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4
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12
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20
24
28
TIME (HOURS)
CONTROL
1 mM cAMP
5 mM cAMP

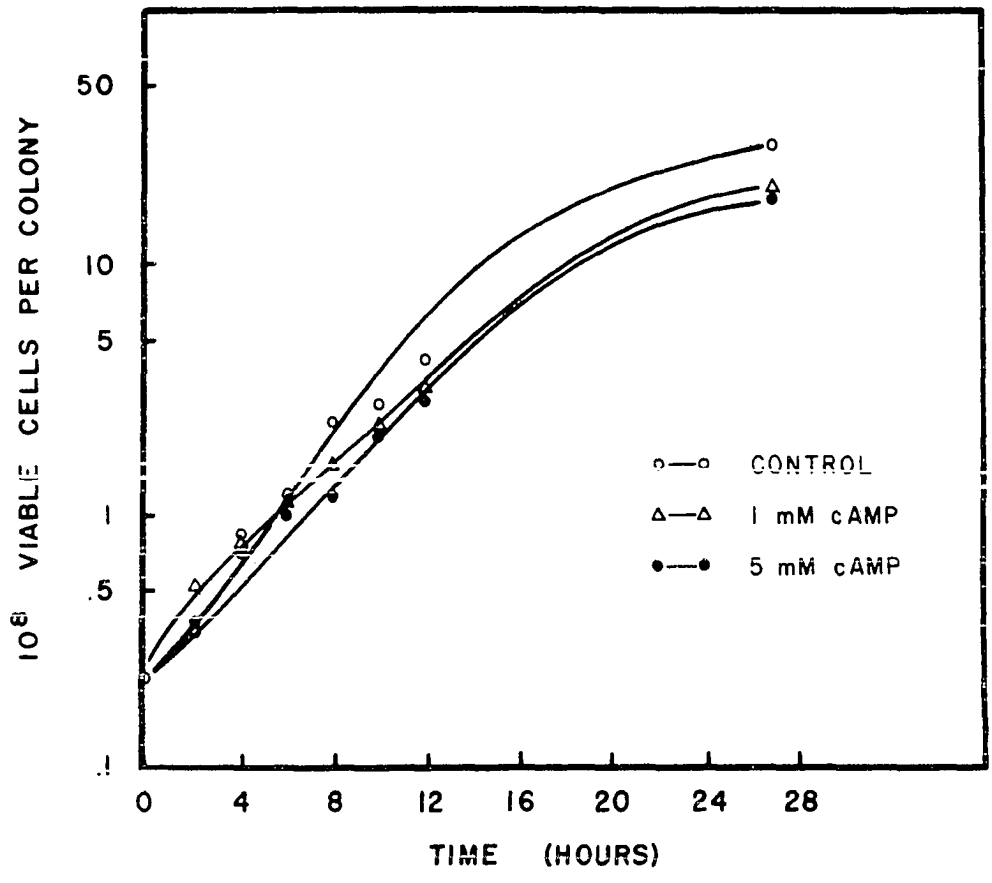
on the growth of IM47 on glycerol agar. Growth was measured by determining the numbers of viable cells in colonies produced from 5-µl broth inocula. The tests of recovery for this method indicated that at least 95% of the viable cells on the agar surface were removed by the washing procedure. From Figure 3 it is apparent that 5 mM cAMP severely inhibited growth of IM47 on glycerol agar, but inhibition did not become evident until about 6 hours after inoculation. At that time viable-cell numbers began to level off then rapidly decreased so that after 27 hours, there were less than 10^4 viable cells remaining in a colony (not shown).

To establish that growth inhibition was not simply a result of cAMP toxicity, glucose was substituted for glycerol as the sole carbon source, and Figure 4 illustrates the results from that experiment. Whereas there may have been some inhibition by the cAMP, when compared with the response on glycerol agar, it was essentially insignificant and may have been a result of experimental error. Comparison of the control cultures in Figures 3 and 4 also indicates that glycerol supported more rapid growth than glucose.

When glucose and glycerol were both present (both at 0.25%) in the synthetic agar medium, a response nearly identical to that on glycerol alone was observed (Fig. 5).

Severe growth inhibition by cAMP was not detected in a glycerol broth medium (Fig. 6). In the experiment illustrated in Figure 6, there was essentially no difference between any of the treatments. In

Fig. 4. The effect of exogenous cAMP upon growth of IM47 on a glucose (0.25%) agar medium at 35 C


50
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10^8 VIABLE CELLS PER COLONY
0
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12
16
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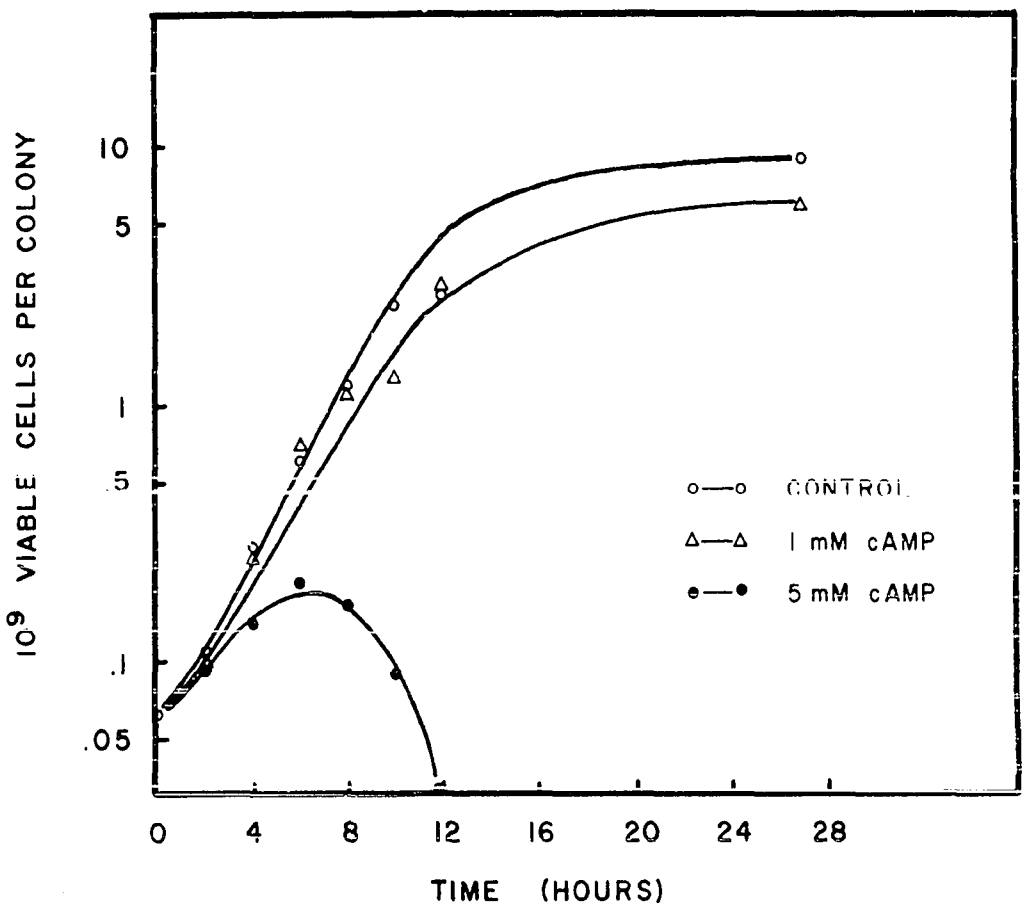
TIME (HOURS)
CONTROL
1 mM cAMP
5 mM cAMP

Fig. 5. The effect of exogenous cAMP upon growth of IM47 on a synthetic agar medium containing glucose plus glycerol (both at 0.25%)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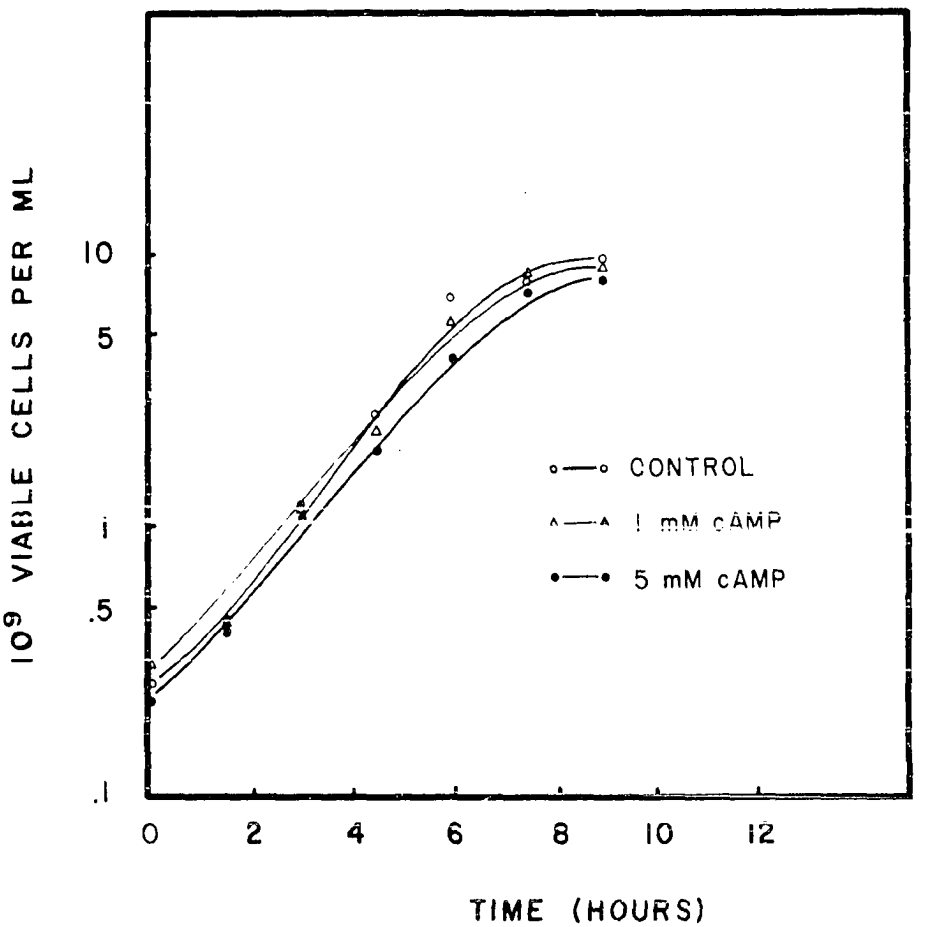
TIME (HOURS)
o—o CONTROL
Δ—Δ 1 mM cAMP
•—• 5 mM cAMP

Fig. 6. The effect of exogenous cAMP on growth of IM47 in glycerol (0.25%) broth at 35 C


10^9 VIABLE CELLS PER ML
10
5
1
.5
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2
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6
8
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12
TIME (HOURS)
CONTROL
1 mM cAMP
5 mM cAMP

subsequent experiments, 5 mM cAMP caused more inhibiton in glycerol broth, but while there was some reduction in the growth rate (doubling time of 75 minutes to 95 minutes), there was no significant difference in the final cell mass. Increasing the concentration of cAMP from 5 to 10 mM had no additional effect (not shown). When glucose was substituted, at the same concentration, for the glycerol, 5 mM cAMP had no effect on either growth rate or final cell mass (not shown).

Five mM cAMP had no significant effect on the growth of Nsw109 on glycerol agar, but when the mutant was grown in mixed culture with IM47, growth of both organisms was inhibited (Fig. 7). These points are also demonstrated, qualitatively, in Figure 8; Nsw109 demonstrated confluent growth on glycerol plus cAMP, except in the area of the streak occupied by IM47 (the vertex), where both organisms were inhibited.

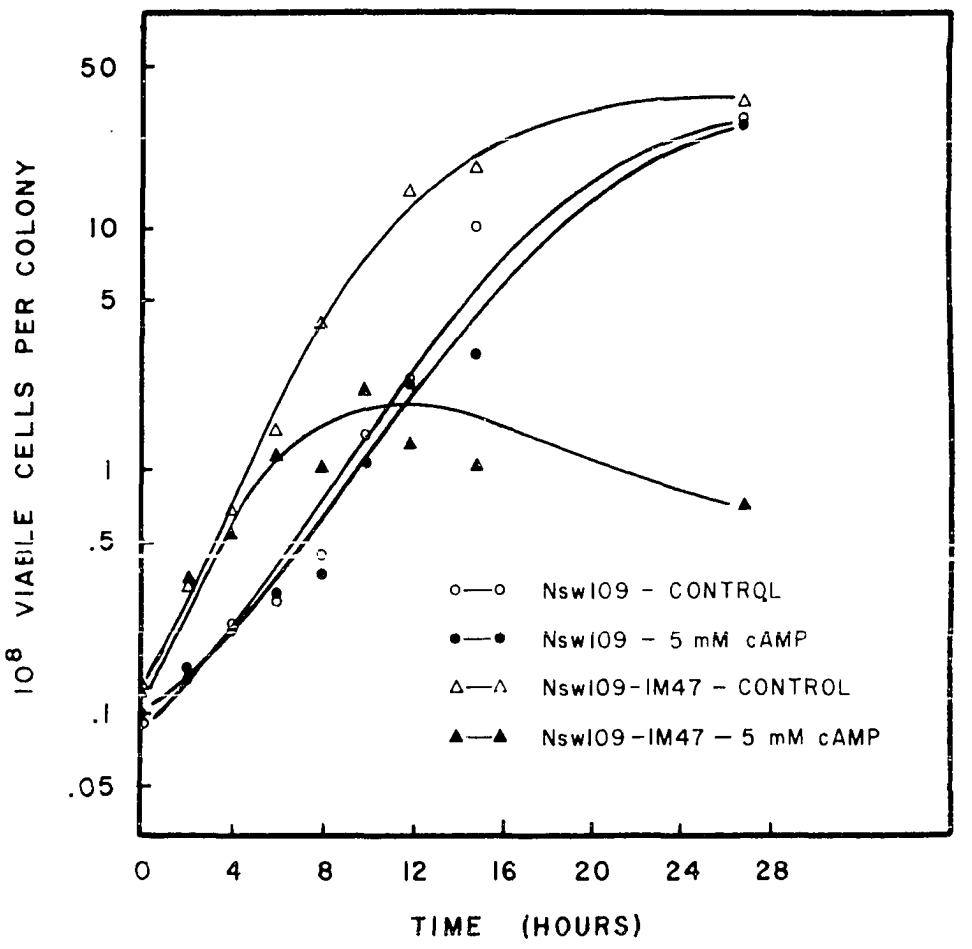
Identification of methylglyoxal in inhibited cultures

cAMP inhibition of growth has been reported previously for E. coli on xylose, arabinose, and glucose-6-phosphate (Ackerman et al., 1974), and for Vibrio parahaemolyticus on starch, dextrin, and glycogen (Iuchi et al., 1975). With E. coli, inhibition was attributed to methylglyoxal, an intermediate in a glycolytic by-pass between dihydroxyacetone phosphate (DHAP) and pyruvate (Fig. 9). Because the response of IM47 resembled that described for E. coli, inhibited cultures of IM47 were examined for the presence of methylglyoxal.

With the thin-layer chromatography system used in this study, authentic methylglyoxal (approximately 0.1 µg spotted) demonstrated

Fig. 7. The effect of exogenous cAMP upon growth of Nsw109 on glycerol (0.25%) agar in pure culture, and in mixed culture with its wild type (IM47)

Approximately equal numbers of mutant and wild-type cells were mixed in suspension for the inoculum in the mixed culture experiment. Viable cell counts for the mixtures are totals of both organisms.


50
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.5
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10^8 VIABLE CELLS PER COLONY
0
4
8
12
16
20
24
28
TIME (HOURS)
Nsw109 - CONTROL
Nsw109 - 5 mM cAMP
Nsw109-IM47 - CONTROL
Nsw109-IM47 - 5 mM cAMP

Fig. 8. The qualitative growth of IM47 (streaked radially in each quadrant) and Nsw109 (streaked perpendicular to IM47) on synthetic agar with 0.25% glycerol (upper left), glycerol plus 5 mM cAMP (upper right), 0.25% glucose (lower left), and glucose plus 5 mM cAMP (lower right)

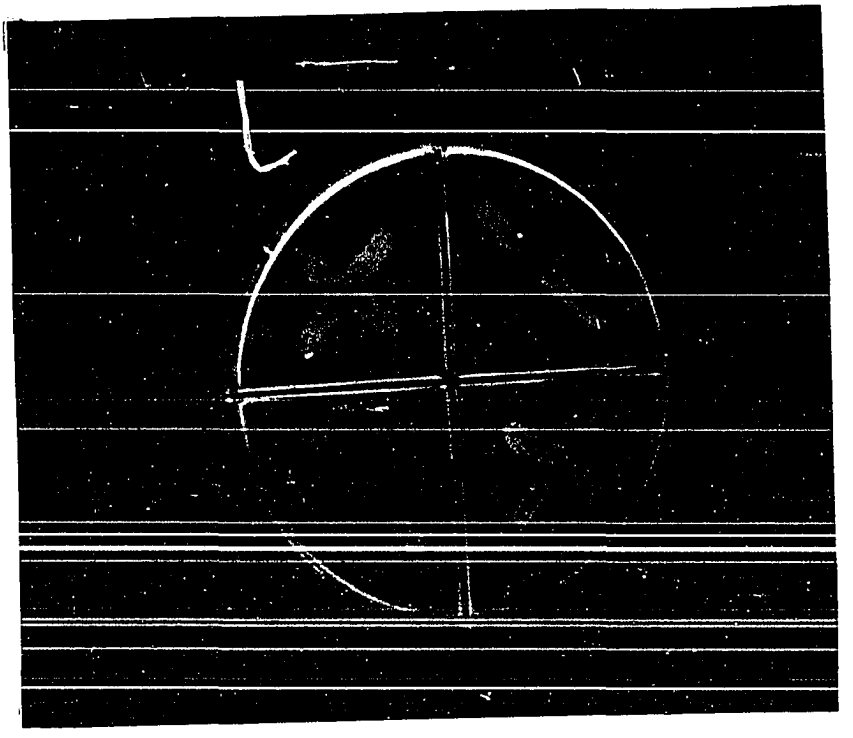
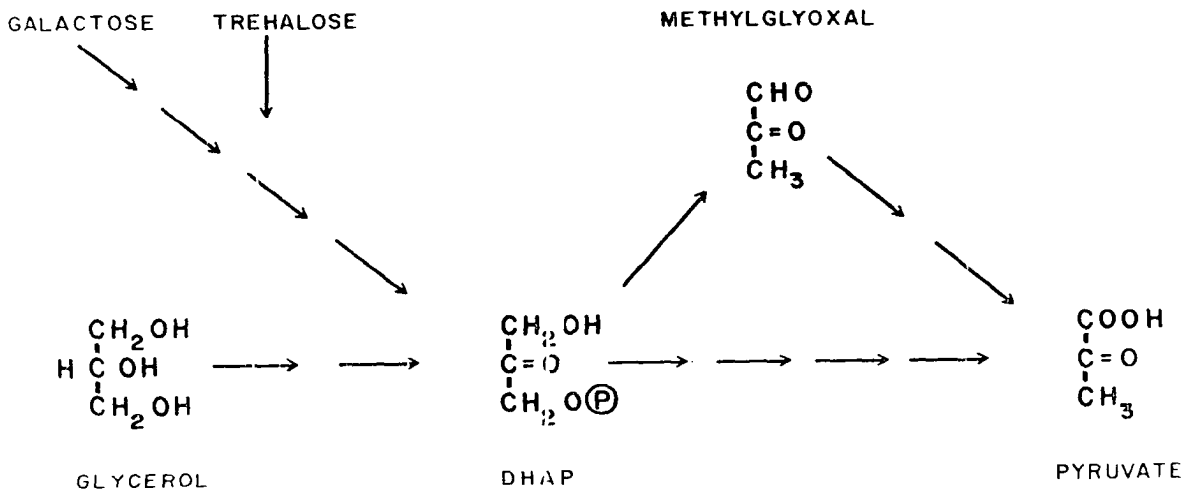


Fig. 9. The structure and relationship of methylglyoxal to related intermediates or substrates that could supply it


GALACTOSE
TREHALOSE
METHYLGLYOXAL
CHO
C=O
CH3
CH2OH
H C OH
CH2OH
CH2OH
C=O
CH2O(P)
COOH
C=O
CH3
GLYCEROL
DHAP
PYRUVATE

two spots: A blue, major spot at an R_f of 0.54 and a very faint blue spot at 0.41. For comparison, glycerol, pyruvate, diacetyl, and glyoxal were also examined in the system. Glycerol and pyruvate failed to produce recognizable spots. Diacetyl (approximately 0.2 µg spotted) produced four spots: A blue, major spot at an R_f of 0.65, and three smaller red spots at 0.46, 0.14, and 0.07. Glyoxal (approximately 0.2 µg) produced two spots: A blue, major spot at an R_f of 0.41 and a smaller blue spot at 0.35.

Cultures (24-hour) of IM47 and Nsw109 on glycerol media, with and without cAMP, were examined for the presence of methylglyoxal. A compound was isolated from cultures of IM47 on glycerol agar plus cAMP that, reacted with DNPH and co-chromatographed with authentic methylglyoxal, reacted in the same way. The compound migrated with an R_f of 0.52 and corresponded to the major spot from methylglyoxal. The compound was not detected in any of the other cultures. When cultures of IM47 were examined at 5 hours, significant amounts of methylglyoxal were detected in both agar and broth cultures with cAMP, and barely detectable amounts were found in control cultures without cAMP.

It was important to know if cAMP produced the same effect on cells in broth as it did in agar. Therefore, resting-cell assays were performed to determine if cells in glycerol broth with cAMP were capable of producing as much methylglyoxal as cells on glycerol agar. Cells of IM47 were harvested from glycerol media, with and without cAMP, at 5 hours post-inoculation, washed, and assayed according to the procedures described. Table 4 summarizes the results from that experiment.

Table 4. Methylglyoxal production by cells from different media

Source of the cells	Specific activity[a]
Glycerol broth - control	103
Glycerol broth - cAMP[b]	164
Glycerol agar - control	95
Glycerol agar - cAMP[b]	160

[a]Specific activity is expressed as ng of methylglyoxal produced per 10^8 cells per minute. Reactions were incubated for 15 minutes.

[b]cAMP was incorporated in the media at 5 mM concentrations.

cAMP binding protein assay

Extracts from IM47 and Nsw109 growing on glycerol broth, glycerol agar, tryptose broth, and tryptose agar were examined with the cAMP binding protein assay. Table 5 summarizes the results from those determinations. At least eight determinations were made on each extract (four sample determinations and four background determinations), and the percent standard error for these determinations averaged less than 5%. The background counts depended on the amount of protein in the sample; for an assay with 1 mg of protein, the background averaged about 5000 c.p.m. With this procedure, a significant amount of precipitate formed in the scintillation vials after the counting cocktail was added. The precipitate was caused by residual $(NH_4)_2SO_4$ in the sample, but it settled to the bottoms of the vials during equilibration and didn't appear to affect the counting efficiency of the system.

Table 5. Specific activities of the cAMP binding protein from IM47 and Nsw109

Source of the extract	Specific activity[a]
IM47 - glycerol broth	1.86
IM47 - glycerol agar	1.29
IM47 - tryptose broth	3.57
IM47 - tryptose agar	8.00
Nsw109 - glycerol broth	0.73
Nsw109 - glycerol agar	1.94
Nsw109 - tryptose broth	7.48
Nsw109 - tryptose agar	10.10

[a]Specific activity is expressed as pmoles of cAMP bound per mg of protein in the extract.

Because of the large number of samples involved, only two extracts could be assayed at one time. To determine what the variation was in samples prepared on different days, four independent trails were performed with IM47 in tryptose broth. The specific activities determined on the four extracts were: 5.13, 7.10, 4.89, and 3.57 pmoles per mg of protein.

Isolation of mutants defective in substrate utilization

Attempts to isolate mutants that were defective in the utilization of specific substrates were not successful. When the mutagenized suspension was plated on nutrient agar and the isolates subsequently

tested on glucose and trehalose, a number of mutants was isolated that failed to grown on either carbohydrate in synthetic media. They did utilize these substrates in fermentation media, and all were able to swarm. When the mutagenized suspension was plated on glucose and the isolates then tested on trehalose, all of them grew. Over 2000 isolates were examined in this way, and no mutants were isolated that would grow on glucose but not on trehalose.

DISCUSSION

A fundamental question regarding the swarming response of Proteus, and one that has direct implications on theoretical considerations of its basis, is whether swarming occurs in natural environments. If it does, and there is no experimental evidence to contradict this assumption, then it should provide the organism with a distinct survival advantage by enabling it to spread on substrates with insufficient surface moisture to allow normal motility. As such, the formation of swarm cells may represent a unique form of morphogenesis that is responsive to changes in the immediate cell environment. These changes could include accumulations of toxic metabolites, the depletion of available energy sources, or a combination of the two.

A popular notion has been that toxic metabolites are responsible for swarm-cell formation, but this interpretation was based largely on the similarity between swarm cells and filamentous cells induced by damaging treatments. Furthermore it does not account for the characteristic flagellation of swarm cells, and its argument is weakened even more if the survival rate of swarm cells is as high as Hoeniger (1964) believed. In a review of cell-division control, Slater and Schaechter (1974) indicated that nearly any chemical, at some concentrations, could cause filament formation, and they noted that in some species, pantoyl lactone restored the ability of these induced filaments to divide. The fact that this compound failed to inhibit swarming (Table 1) is certainly not proof that swarm cells are formed by a different mechanism, but

when all of the available information is considered, there is no more evidence for the theory that swarm cells are formed by the nonspecific action of toxic wastes than there is for other theories. An alternative role for the toxic waste products might involve their specific regulation of certain gene functions, responsible for swarm-cell formation; they may act as inducers or derepressors. Perhaps the biggest objection to this explanation is that it would involve genetic regulation by compounds quite different from the compounds more commonly associated with these regulatory systems.

Another alternative, and one that has some theoretical and experimental support, is that swarm-cell formation is genetically controlled and is induced by the depletion of energy sources in the medium. The essence of this hypothesis is that there are specific genes that are activated by a reduction in the energy level of the cell. In catabolite repression, we have such a system, and the available evidence indicates that it is mediated by cAMP. Catabolite repression is a mechanism that apparently has evolved to improve the survival ability of an organism by controlling cell functions that are required only under certain conditions; the formation of swarm cells might be considered as such a function. At least two essential characteristics are associated with swarm-cell formation, excessive flagellation and extreme cell length. If flagella synthesis and cell septation were regulated by catabolite repression, then their expression would be indirectly controlled by the nutritional condition of the cell environment. It is of particular interest, therefore, that in a number of genera, flagella synthesis is

subject to catabolite repression (Iuchi et al., 1975). It remains to be shown, however, that flagellation is controlled similarly in swarming-species of Proteus.

The rationale that was followed in developing a new working hypothesis to explain the formation of swarm cells can be summarized in the following proposed sequence of events:

1. The cells in a colony on an agar surface deplete the available nutrients in their immediate vicinity, and the energy level of the cell drops.
2. The reduction in the cellular energy level is reflected in an elevated level of cAMP.
3. The cAMP binds, with the cAMP receptor protein, to specific points (promoter sites) on the bacterial chromosome and facilitates RNA polymerase binding and subsequent transcription of the adjacent DNA. Expression of these regions (individual genes or operons) results in rapid synthesis of flagella and inhibited cell septation; swarm cells are produced.
4. The swarm cells move across the agar surface onto fresh medium where there is an increased supply of available nutrients.
5. The elevated level of available nutrients raises the energy level of the cell and reduces the cAMP level. This in turn results in repression of the gene(s) responsible for

swarm-cell formation, and the swarm cells revert back to the short forms.

This is a very simplified description which leaves innumerable unanswered questions; however, it provided a working hypothesis that could be approached experimentally.

If swarm-cell formation is controlled by catabolite repression, one would predict that repression of swarm-cell formation could be overcome by the addition of exogenous cAMP. The results of this study indicate that added cAMP will not bring about swarm-cell formation under conditions that fail to support normal swarm-cell formation. However, the addition of cAMP to a system where swarming occurs normally brings about premature swarming (Table 1). The concentrations of cAMP, required to produce this effect, are not significantly different from those that have been used in other systems to overcome catabolite repression (Coppola et al., 1976).

An alternative approach would be to reduce the intracellular concentration of cAMP and examine its effect on swarming. One would predict that maintaining a reduced level of cAMP would inhibit swarm-cell formation. The classical method for reducing cAMP levels has involved the addition of certain carbon sources, such as glucose or pyruvate, to the medium (Perlman and Pastan, 1969). The addition of these compounds at a final concentration of 0.5% to tryptose agar had no effect on swarming of IM47. These results are difficult to interpret because glucose and pyruvate have not been shown to act as repressors

in P. mirabilis. Chemotaxis in E. coli in tryptone soft agar is inhibited by the addition of glucose, gluconate, and mannitol (Silverman and Simon, 1974), but, because IM47 fails to utilize gluconate and mannitol and grows more slowly on glucose than it does on glycerol, one would not expect these compounds to act as efficient repressors. It is not surprising, therefore, that these compounds had no effect on swarming or chemotaxis of IM47. The situation is complicated further by the fact that a colony on an agar surface is not a homogeneous system, and the cells within are not all exposed to the same environmental conditions. This may be the best explanation for the observation that not all cells in a colony form swarm cells (data not shown).

Recently, Coppola et al. (1976) reported that a cytokinin (N^6-[Δ^2-isopentenyl] adenine), at 10^{-3} mM concentration, overcame the effects of exogenous cAMP on growth and β-galactosidase synthesis in E. coli, and they speculated that it affected the degradation of cAMP by a phosphodiesterase. The addition of kinetin (a related compound) to tryptose agar appeared to inhibit, slightly, swarming of IM47, but this effect was observed only at the comparatively high concentration of 5 mM (Table 1). That kinetin produces the same effect as the cytokinin in E. coli has not been shown.

The results from studies with the nonswarming mutants provide convincing evidence for the involvement of cAMP in swarming. The most direct evidence is the ability of exogenous cAMP to stimulate a swarming response in Nsw203, Nsw214, and the two revertants isolated from Nsw214 (Table 2). Unfortunately, the lesions in these strains

that are overcome by the cAMP could not be defined. The phenotypes of Nsw203 and Nsw214 differed significantly in the absence of cAMP (Tables 2 and 3), but they demonstrated similar swarming responses in its presence. With Nsw214, the pattern of carbon-source utilization and the stimulation of swarming suggested that it might be defective in adenyl cyclase, but this possibility seems remote because cAMP did not stimulate growth on any of the carbon sources that Nsw214 could not ordinarily use. These results might be explained as the presence of multiple lesions induced by the nitrosoguanidine, but the pattern and frequency of reversions and the characteristics of the revertants suggest that the defects are all related to a common lesion. A reversion to swarming did not enable the organism to demonstrate normal chemotaxis; conversely, a reversion to chemotaxis did not enable the organism to swarm (it would swarm in the presence of exogenous cAMP). In addition, a reversion to pyruvate utilization did not enable the organism to use trehalose. The reversion rates for all of these characteristics were very high. Despite the fact that these results resist simple explanation, the stimulation of swarming by cAMP is reproducible, and, with the revertants, is very strong. This is the first description of chemical-dependent swarming by a mutant of Proteus. Gaydos et al. (1972) reported that a number of short-chained organic acids induced swarming of nonswarming mutants of P. mirabilis, but they continued to swarm in the absence of the inducing compounds. Ulitzur (1975) has described mutants of Vibrio alginolyticus that were induced to swarm by by-products of other mutants and by certain chemicals at

low concentrations. This report is interesting because the morphological changes associated with swarming of certain Vibrio strains are analogous to those of Proteus, and one would expect the mechanisms involved to be similar also. The previous study from our laboratory (Williams et al., 1976) was very similar to that of Ulitzur, including the methods of mutagenesis and mutant selection and the tests of mutant swarming, but whereas Ulitzur isolated several nonswarming mutants that responded to certain chemicals, none of the mutants we isolated demonstrated a similar response. The results from the two studies suggest that the mechanisms involved are different, but until the actual mechanisms are confirmed, their apparent contradiction is concerning.

A second class of mutants examined in this study provided indirect evidence for the involvement of cAMP in swarming. The mutants in this group (Nswl09, -221, and -228) were resistant to the inhibitory effects of cAMP on a glycerol agar medium. Growth inhibition of IM47 in this system appeared to be caused by a toxic accumulation of methylglyoxal. The cAMP probably stimulated a rate-limiting step in the utilization of glycerol that resulted in an excess of DHAP. This, in turn, served as substrate for methylglyoxal synthetase (the enzyme that converts DHAP to methylglyoxal) and caused a toxic accumulation of methylglyoxal. This interpretation is consistent with the findings of Freedberg et al. (1971). They found that a mutant of E. coli, constitutive for glycerol utilization and possessing a glycerol kinase that was insensitive to feedback inhibition by fructose diphosphate, accumulated toxic levels

of methylglyoxal when grown on glycerol. If glycerol utilization is sensitive to catabolite repression in P. mirabilis, then exogenous cAMP might overcome its normal regulation and result in methylglyoxal toxicity. This could also explain the inhibition of growth on galactose and trehalose, if the utilization of these substrates is also subject to catabolite repression. The failure to demonstrate severe inhibition in glycerol broth may reflect a difference in the concentration of accumulated methylglyoxal. Resting-cell assays with IM47 indicated that cells from glycerol broth with cAMP were capable of producing the compound in quantities similar to those of cells from an agar surface (Table 4). Because of the density of cells on an agar surface and the reduction in diffusion rate by agar, however, excreted metabolites will probably accumulate in higher concentrations on agar than in broth, where free diffusion can occur. The fact that significant quantities of methylglyoxal were produced in the resting-cell assays by cells from glycerol media without added cAMP indicated that the methylglyoxal by-pass may fulfill an important metabolic function under certain growth conditions.

The fact that three nonswarming mutants were resistant to inhibition by cAMP is presumptive evidence that the system of regulation, mediated by cAMP, is somehow involved in swarming. Ackerman et al. (1974) described mutants of E. coli that were insensitive to cAMP on xylose and designated the mutation as cxm for cAMP-xylose-methylglyoxal production. The cxm mutation did not protect the cells from inhibition by cAMP on arabinose. The mutations in Nsw109, 221, and 228 differed

from the cxm mutation because they protected the cells from inhibition on trehalose as well as glycerol. The mutations may be in the synthesis of methylglyoxal synthetase or in a common regulatory step in the utilization of methylglyoxal-yielding carbon sources. The results from the binding protein assays (Table 5) indicated that the activity of the cAMP receptor protein was normal in Nsw109. This is consistent with the fact that this mutant was able to utilize the same carbon sources that its wild type did. The inability of these mutants to swarm and their resistance to cAMP inhibition might be caused by the presence of an overactive phosphodiesterase which might prevent the intracellular-cAMP concentration from reaching levels necessary to activate both processes. An alternative explanation for these mutants would be the presence of multiple lesions induced by the nitrosoguanidine.

By no means do the results from this study prove the hypothesis that cAMP is responsible for the regulation of swarming, but they do provide presumptive evidence that it is involved. The essential question, whether or not fluctuations in the cAMP concentration are responsible for controlling the response, remains unanswered. Because cAMP is necessary for flagellation in other organisms (Iuchi et al., 1975), one would predict that it is also necessary for swarming of Proteus. The isolation of mutants of P. mirabilis that are defective in adenyl cyclase or the cAMP receptor protein should provide definitive evidence. In addition, mutants of these types would provide additional information about the relationship between flagellation and cell elongation. The working hypothesis described previously did not specify

exactly how these two characteristics might be controlled. If cAMP is responsible for regulating swarming, its mechanism of control of flagella synthesis is probably similar to that in other organisms, but how it might control cell septation is completely open to speculation. The mechanisms responsible for controlling septation are still poorly understood; some steps in septum formation are dependent, however, on specific gene products (Walker et al., 1975). One could postulate that other proteins, regulated by catabolite repression, might interfere with the proteins necessary for septum formation.

Alternately, the inhibition of septation might be a function of flagella synthesis, and derepression of flagella synthesis automatically leads to filament formation. Jones and Park (1967a) speculated that uncontrolled flagella synthesis inhibited septum formation by depleting the intracellular amino acid pool. In a previous study from this laboratory involving the isolation and characterization of nonswarming mutants, the majority of nonmotile mutants also failed to form elongated cells (Williams et al., 1976). This fact suggests a specific relationship between flagella synthesis and cell elongation, but if elongation is only a function of a depleted amino acid pool, one would expect that even the synthesis of defective flagellar proteins would cause cell elongation. Another explanation might be that the physical presence of flagella, inserted in the cell membrane or envelope, inhibits septum formation by blocking specific sites. Thus, any mutation that affected the synthesis or insertion of the flagellar apparatus in the cell

membrane and envelope would also affect cell elongation. In any case, there are several possible ways that flagella synthesis and septum formation could be coordinately controlled so that elevated levels of cAMP could trigger the formation of swarm cells.

Even if the working hypothesis (that fluctuations in the level of cAMP are responsible for controlling swarm-cell formation) described earlier is correct, several questions remain unanswered: Sodium chloride is normally required for swarming (Naylor, 1964), and in salt-deficient media, swarm cells are not formed (data not shown). How does sodium chloride act to support both cell elongation and flagella synthesis? PNPG also inhibits swarm cell formation (Hoffman, 1974), but it has no effect on motility or growth (Williams, 1973). Swarm cells will migrate on a non-nutrient medium (Williams et al., 1976), but what is the energy source that supports their movement, and is it unique to swarm cells? There is evidence that an extracellular slime is present during swarming (VanderMolen and Williams, circa 1976), but is its presence necessary and/or unique to swarming? Smith (1972) described the inhibition of swarming by the incorporation of charcoal in the medium, and he considered the possibility that the charcoal adsorbed cAMP. He discounted the possibility after failing to detect stimulation of swarming from the addition of cAMP to nutrient agar, but because he failed to describe his experimental methods, it is impossible to compare his results with the results from my study. One of the most perplexing and perhaps important questions is: Why are swarm cells formed on an agar surface but not in broth? A possible clue to the answer for this question was

provided by the results from the binding-protein assay (Table 5). In three of the four cases, extracts from cells, grown on agar surfaces, had significantly higher activities than those from cells grown on the corresponding broth media. Perhaps more significantly, extracts from cells grown on complex media had higher specific activities than those from cells grown on synthetic media. In E. coli, a synthetic glycerol medium supported good motility (Adler and Templeton, 1967), but the same type of medium supported very poor motility with IM47. Admittedly, the results from the binding-protein experiments were preliminary and require confirmation, but it is tempting to speculate that flagellation and perhaps swarm-cell formation are controlled, not only by the level of cAMP, but also by the activity of the cAMP receptor protein. This interpretation could explain a number of observations concerning flagellation and swarm-cell formation. At the very least, these results suggest that further investigations of environmental and physiological factors that affect motility of P. mirabilis would be worthwhile.

SUMMARY

The critical event in the swarming response of P. mirabilis appears to be the formation of swarm cells; once they are formed, their movement across the agar surface is nonchemotactic and is probably a consequence of their extreme length and excessive flagellation. The results from this study provide reasonable evidence that cAMP is involved in the morphogenesis of swarm cells. The evidence consists of the findings that: The addition of 5 or 10 mM cAMP to a medium that normally supports swarming results in premature swarming. Exogenous cAMP induces swarming responses by nonswarming mutants, and these responses are dependent on the continued presence of the cAMP. Some mutants of P. mirabilis, isolated as nonswarmers, are resistant to the inhibitory effect of cAMP on synthetic agar media, containing certain carbon sources.

cAMP by itself, however, is not sufficient to induce swarm-cell formation in a system where it does not occur normally. This fact, along with observations of medium requirements necessary for motility, suggest that there may be at least two systems responsible for regulating flagella synthesis; only under certain circumstances, do these systems interact to trigger the formation of swarm cells. The results from the binding-protein assays suggest that the level or activity of the cAMP receptor protein is variable and may depend on cultural conditions. If this is true, then swarm-cell formation may depend on an elevated level of the cAMP receptor protein (which is controlled

by cultural conditions) and an elevated level of cAMP (which depends on the immediate energy state of the cell). In this way, the formation of swarm cells would be triggered only under conditions where levels of both cAMP and cAMP receptor protein are elevated.

By itself, the fact that cAMP is somehow involved in swarm-cell formation implies that the response is genetically controlled and as such, represents a unique form of morphogenesis. Regardless of the findings of this study, it is surprising that more attention has not been devoted to this phenomenon, not only from the standpoint of control of morphogenesis but also with respect to control of cell division.

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